VANM212.001AUS

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Remacle, et al.
Appl. No.	:	09/816,763
Filed	:	March 23, 2001
For	:	METHOD AND KIT FOR THE SCREENING, THE DETECTION AND/OR THE QUANTIFICATION OF TRANSCRIPTIONAL FACTORS
Examiner	:	Kim, Young J.
Group Art Unit:		1637

DECLARATION UNDER 37 C.F.R §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. This Declaration is being submitted to demonstrate the advantages resulting from use of spacers between about 50 and about 250 bp in length and the lack of these advantages in the methods described in the cited Peterson and Saiki references.

2. I am an inventor on the above-identified patent application and am familiar with the specification and prosecution history.

3. I have extensive experience in the field of the claimed invention as indicated in the attached Curriculum Vitae provided herewith as Exhibit A.

4. In the claimed methods, a double stranded DNA sequence comprising a binding site for transcriptional factor(s) is connected to the surface of a solid support by a spacer of between about 50 bp and about 250 bp in length. We conducted experiments to assess the dependence of the detection of transcriptional factors on microarrays on the size of the spacer.

Exhibit 1A attached hereto shows the signals resulting from NFκB binding to recognition sites linked to spacers of 6, 20, 50 or 100 bp. Exhibit 2A (attached hereto) shows a quantitation of the results shown in Exhibit 1A. Extracts were obtained either from unstimulated cells or cells stimulated with IL-1. In Exhibit 1A, an NFκB signal increase in stimulated cells (WI38 + interleukin-1) was observed compared to non stimulated cells (WI38). This specific signal was observed with all the spacer sizes tested, which range from 6 bp (close to the conditions used by Peterson et al., were the spacer is 8 bp long) to 100 bp. To quantify these saturating signals, we had to perform the fluorescence scanning at a lower gain (80 instead of 100). Figure 2A provides a quantitation of the results in Figure 1A.

Exhibit 1B attached hereto shows the signals resulting from ELK-1, C-MYC, STAT1 and STAT3 binding to recognition sites linked to spacers of 6, 20, 50, or 100 bp. Exhibit 2B (attached hereto) provides a quantitation of the results shown in Exhibit 1B. Contrary to detection of NFκB factor which was still detectable with the use of a small spacer, some factors were hardly detectable (STAT3), or not detected at all (Elk-1) with a small spacer. We also found that the signals measured with spacers below 50 bp may not increase linearly with the spacer size (see STAT3 data in Exhibit 2B).

Exhibit 3 compares the results obtained with the NFκB, ELK-1, C-MYC, STAT1 and STAT3 binding sites linked to 6 bp spacers in Exhibits 1. The high variability in signal detection using 6 bp spacers is totally incompatible with the simultaneous analysis of more than one factor. However, in microarrays such as those used in the presently claimed methods, it is important to be able to detect binding of many transcription factors simultaneously.

Exhibit 4, attached hereto, shows the extent of NFκB binding to its recognition site linked to spacers of 6, 50, 100, 150, or 250 bp. We observed an inhibition of the binding when the spacer size exceeds 250 bp, probably due to steric hindrance reason.

As illustrated in the accompanying Exhibits, spacers between 50 and 250 bp in length provide significantly enhanced signal levels relative to those obtained with spacers of 6 bp. This is important in the context of a microarray because the binding conditions for all of the factors to be assessed on the array will be uniform while the optimal binding conditions for each factor are different. In the foregoing experiments, optimal binding conditions were used for each of the factors being assessed. However, enhancing the signal levels using spacers of the lengths recited in the claims enhances the ability to evaluate the binding of multiple factors under uniform conditions.

5. Peterson et al. (WO95/30026) describes assays for screening for drugs which interfere with sequence-specific protein-DNA binding. Peterson et al. neither suggest nor mention the use of a spacer. However, for the sake of argument, the DNA sequences preceding the protein binding sites in their examples may be considered as such. These short sequences, which are 10 bp in average, are, however, not long enough to be applied to microarray experiments. As discussed above, the use of short spacers significantly reduces signal levels relative to those obtained using spacers of the lengths recited in the claims. Furthermore, as discussed above, such short spacers are incompatible with methods in which the binding of multiple factors to a microarray is assessed using uniform binding conditions.

6. Saiki et al. (WO 89/11548) teach an improved nucleic acid hybridization assay reagent, which uses a single-stranded (ss) DNA tail in order to move the hybridization region of a probe away from the support and thereby improve the hybridization efficiency between two single-stranded nucleic acid sequences. Saiki et al. neither suggest nor mention the use of a double-stranded (ds) DNA spacer connected with a specific sequence being able to bind one or more transcriptional factors or any other protein.

The kinetics and nature of ssDNA-ssDNA binding is completely different from the kinetics and nature of binding of a transcription factor to a double-stranded target sequence. The binding mode of ssDNA-ssDNA is in no way comparable to that of transcriptional factor-dsDNA, due to the completely different nature of the interacting partners. First, DNA and proteins are different classes of molecules having different physical and chemical properties. While ssDNA is made of a succession of 4 types of nucleotides which form a uniform molecule with a well defined structure when present in double-stranded form, proteins are made of 20 different amino acids and exhibit a large variety of properties (hydrophilic-hydrophobic, acido-basic, ...), according to their composition and their possible post-translational modifications. This is of particular importance in the context of transcriptional factors, as many of them require phosphorylation or association events to become functionally active and acquire/retain a DNA-binding capacity. Accordingly, each transcriptional factor exhibits unique structural and therefore binding properties that are very different for one another. The complexity of transcriptional factor-dsDNA interactions is further increased by the absolute requirement for the factor to exhibit its native conformation. Indeed, the functionality of the DNA binding motifs of transcriptional factors highly depends on their surrounding structural context.

The nature and kinetics of the interactions between ssDNA-ssDNA and transcriptional factor-dsDNA are also different. The ssDNA-ssDNA interaction is mediated through hydrogen bonds between the nucleotide bases of each strand that are favored at high temperature and salt concentration. Transcriptional factor-dsDNA interactions are mediated by much more complex interactions, involving salt bridges, hydrogen bonds, hydrophobic interactions and metallic ion chelation, and must take place at moderate temperature to avoid the denaturation of the protein. Another major difference resides in the size of the interacting partners. While ssDNA molecules exhibit some structural flexibility which allows the interaction of any internal sequence with an immobilized complementary sequence, the globular nature of transcriptional factors as well as their important size render their interaction with dsDNA target sequences much less flexible.

Because the kinetics and complexity of hybridization of single-stranded nucleic acids are vastly different than protein-DNA interactions, one skilled in the art would not look to Saiki to provide guidance on assessing protein-DNA interactions.

Furthermore, Saiki did not teach or suggest that spacers in the size ranges of about 50 to about 250 bp are preferred. In fact, the preferred embodiment of Saiki et al. employs tails of at least 400 nucleotides in length (page 17, line 24 and examples). Moreover, in Saiki et al. "the exact number of nucleotides is not critical" (page 17, lines 25-26), and "larger tails are doubly preferred" (page 18, lines 10-11).

In addition, the nature of the tails used by Saiki et al. also does not suit the method described in the present invention. Saiki et al. use ssDNA tails, which are preferably composed of repeated nucleotides (homopolymers; page 17, line 13 and examples), and argue that the use of heteropolymeric tails may be a problem (page 17, lines 31-32 to page 18, lines 1-4).

Furthermore, the homopolymer ssDNA tail of Saiki et al. is synthesized by enzymatic reaction. The terminal transferase (Tdt) tails the probe at the 3' end with dTTP (page 24, line 20). This method is not compatible with the synthesis of dsDNA spacer as required in the present invention.

Finally, Saiki et al. suggest that diverse sets of oligonucleotides specific for different sequences can be immobilized on the same membrane (page 24, lines 3-5). However, the Example 1 of Saiki et al. shows that the array is not formed in a miniaturized format since 100 µl of probe are spotted onto the filters. This volume is about 10^5 times higher than the volume currently delivered on microarrays and will not allow reaching a density of at least 4 spots/cm^2 of solid support surface as in the present invention, because of the solution spreading. It is important to note that the kinetics of hybridization would differ significantly between a macroarray and a microarray due to the different amount of immobilized probe. This further highlights the difference between the present invention and the method of Saiki et al.

Thus, the nature of the problem addressed by Saiki (nucleic acid hybridization) is vastly different than the assessment of protein-DNA interactions. Furthermore, Saiki does not teach or

suggest that double-stranded spacers would have the advantages for assessing protein-DNA interactions which are achieved by the present invention.

7. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: 28 September 2004 By: _____
Jose Remacle

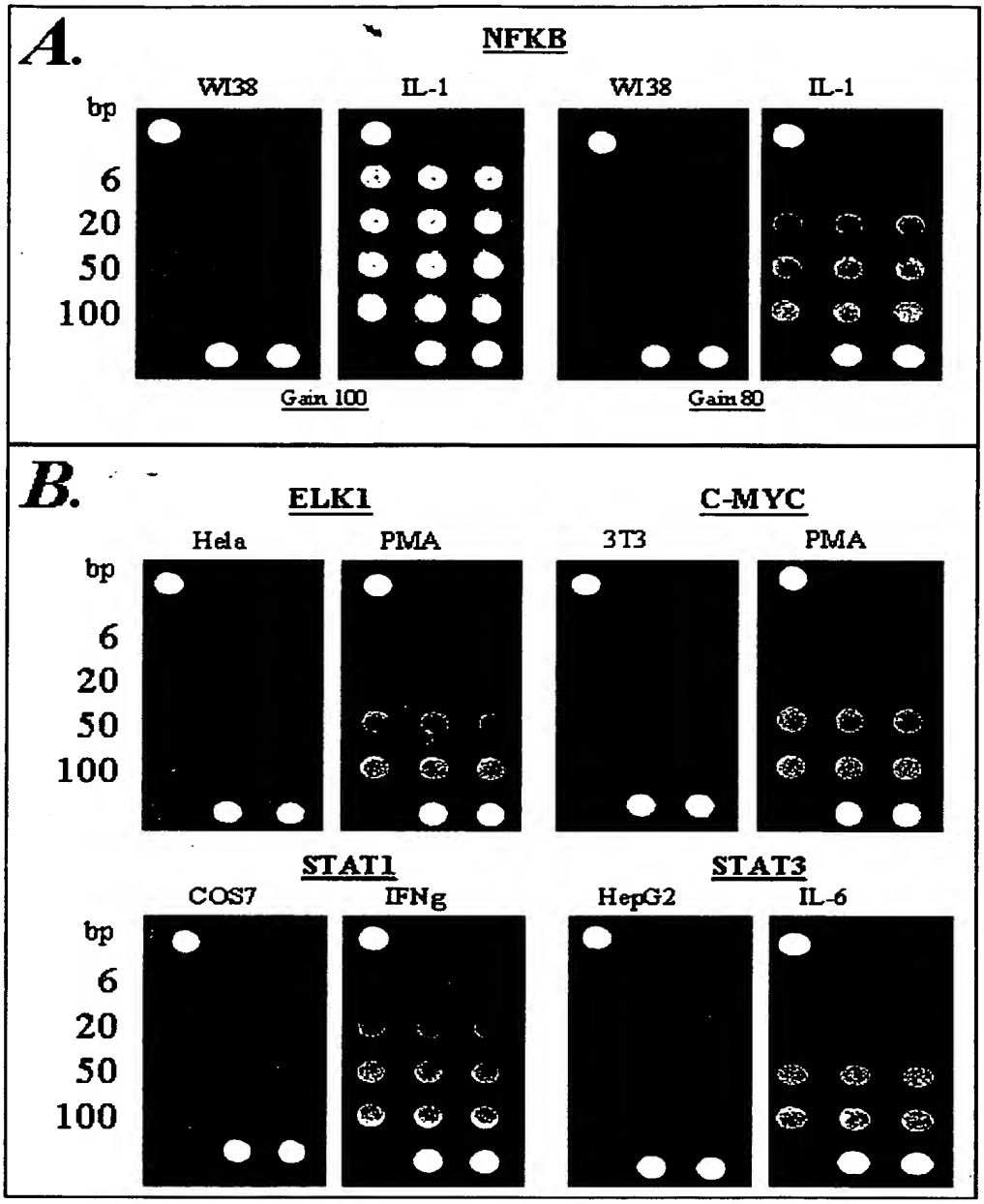
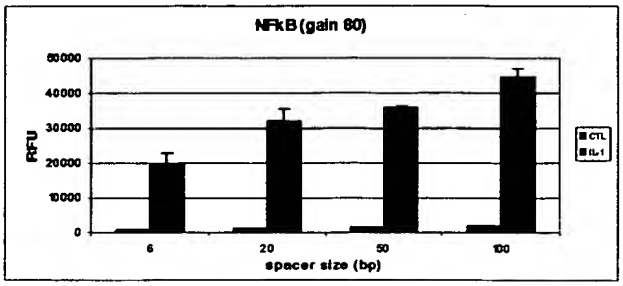
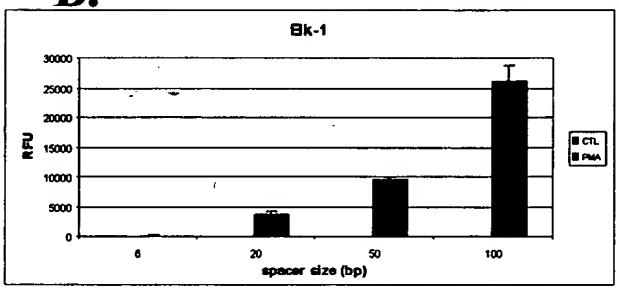


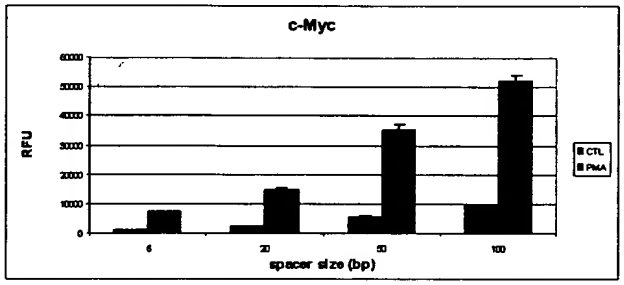
Exhibit 1: detection of transcriptional factors (TF) on microarrays. Spots are present in triplicate; top left and bottom right spots are positive controls; DNAs corresponding to each TF comprise a specific binding site and a common spacer of increasing length, specified by the numbers on the left of each array. Signals for each TF were measured for control (left array) and stimulated (right array) cells in each factor's optimal assay conditions: NFκB: WI38 + interleukin-1 (IL-1); Elk-1: HeLa + phorbol 12-myristate 13-acetate (PMA); c-Myc: NIH3T3 + PMA; STAT1: COS7 + interferonγ (IFNγ); STAT3: HepG2 + interleukin-6 (IL-6). Signals were obtained with Cy3-labeled secondary antibodies and fluorescent scanning using a ScanArray Express microarray scanner from Packart BioScience and a laser power of 100. Scans were performed with a gain = 100, except for NFκB, where a gain = 80 was also used.

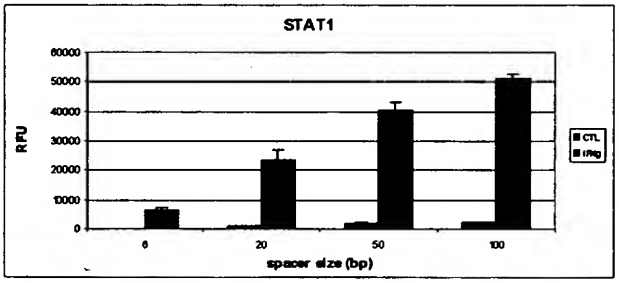
A.



B.







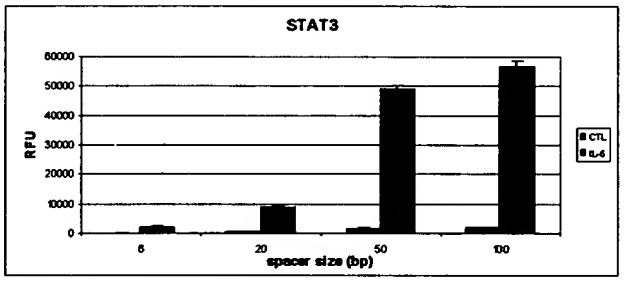


Exhibit 2: quantification of signals from figure 1. Y axis is in relative fluorescence units. Scanning was performed with a gain = 80 (A: NFκB) or 100 (B: Elk-1, c-Myc, STAT1, STAT3) using a ScanArray Express microarray scanner from Packard BioScience and a laser power = 100.

■ : non-stimulated cells; ■: stimulated cells.

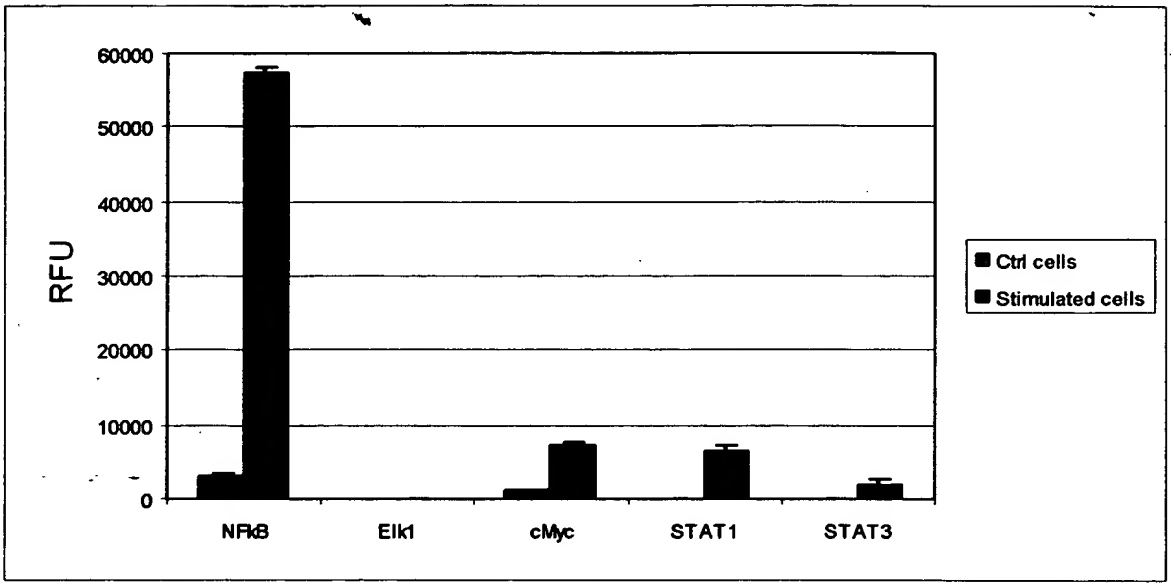


Exhibit 3: comparison of the fluorescence signals from figure 1 measured at a gain = 100 with a spacer of 6bp.

■ : non-stimulated cells; ■ stimulated cells.

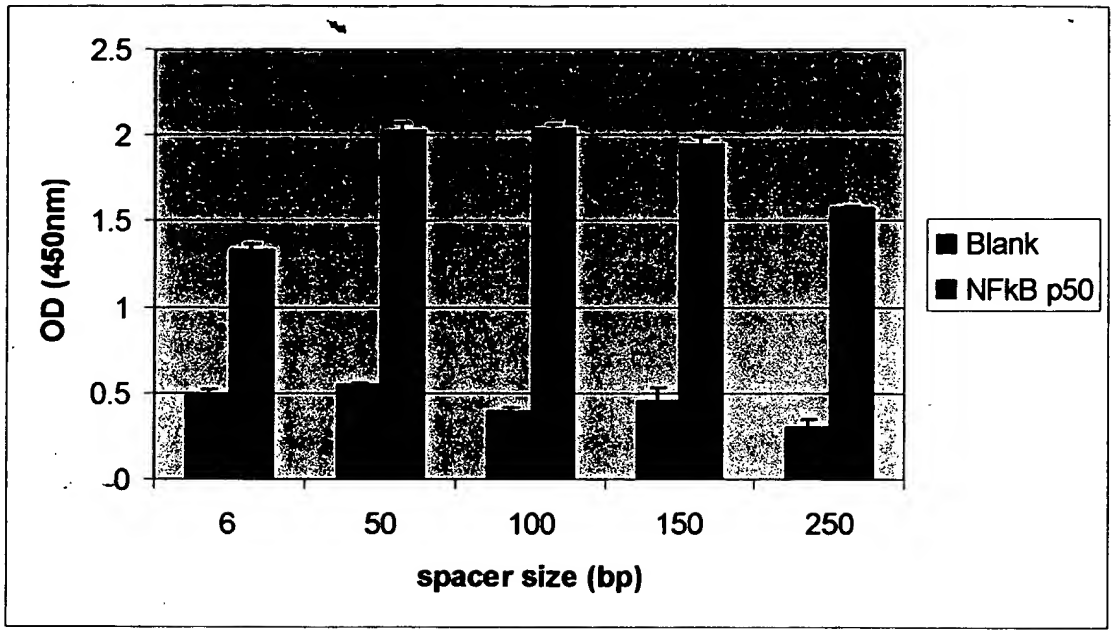


Exhibit 4: NFκB (p50) signals measured in multiwell assays using spacers of increasing size. 0.4μg of purified p50 was used per well.

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Exhibit A: Short CV

CURRICULUM VITAE

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DEGREES

Bachelor of Chemistry with maxima cum laude, 1970
Université Catholique de Louvain, Belgium.

Ph.D. in Sciences, Biochemistry, with maxima cum laude, 1973
Université Catholique de Louvain, Belgium.
Directeurs de thèse : Profs H. Beaufay and A. Trouet.
Laboratoire de Chimie Physiologique, Prof. C. de Duve.

POSITIONS

1970 - 1971 : *Junior research of the National Fondation for Scientific Research (F.N.R.S.)*
1971 - 1974 : *Research assistant of the F.N.R.S.*
1973 - 1974 : Fellowship of "Belgian American Educational Foundation" (Bourse C.R.B.)
1974 : Research fellowship of the European Molecular Biology Organization (E.M.B.O.)
1974 *Associate professor* Facultés Universitaires Notre-Dame de la Paix, Namur.
1980: *Professor* Facultés Universitaires Notre-Dame de la Paix, Namur
Director of the Laboratory of Cellular Biochemistry.
1985: *Full Professor, with tenure*
1992: *Visiting Scientist* University of Maryland, Baltimore County Campus

AWARDS

1968 : Prix de "l'Union Carbide European Research Associates"
1973 : Bourse William Hallam Tuck, of the fondation Francqui

1984 :Prix Vander Stricht de la Fondation André Vander Stricht.
1992 : Senior Research Scholar at the University of Maryland, Baltimore for 1992-1995.

PROFESSIONAL EXPERIENCE

Research stage at the Rockefeler University, Prof. C. de Duve, from July to September 1973.

Post-doctoral research at the University of California, San Diego, U.S.A., from September 1973 to August 1974, in the laboratory of Prof. S. J. Singer.

- Scientific mission of 4 months at the Biochemical Engineering Department of the University of Maryland in Baltimore,Laboratory of Prof G. Rao, in 1992.

Scientific mission at the Biochemical Engineering Department of the University of Maryland at Baltimore as Senior Research Scolar in March-April 1993.

SCIENTIFIC RESPONSABILITIES :

Head of the laboratory of cellular biochemistry and biology

Actual composition (1997)

7 PhDs in Science full research

12 PhDs Students

8 Graduate full research

8 Under-graduate students

7 technicians

Students and researchers already formed

Director of 16 PhDs Thesis passed from 1981 to 1997.

Director of 74 graduate students from 1974 to 1997.

RESEARCH CONTRACTS

<u>Research Contracts with Industries</u>

30 research contracts with Laboratoires Dausse, Synthélabo, Solvay-Biotec, Compagnie des développements agro-alimentaires (CDA), Kali-Chemie Pharma, La Floridienne, CELAC, laboratoires Oberval, Laboratoires Beaufour, UCB-Pharma, Lambdatech, Lipha, IPSEN, Zyma, Madaus Pharma, Servier.

Scientific Grants and Research contracts

14 contracts with the FNRS, FRFC,IRSIA, and Région Wallonne

PROFESSIONAL AND SCIENTIFIC ASSOCIATIONS

Member of 15 scientific societies

Member of 33 Ph.D. thesis jurys

Member of the research comitte for Biomed 1 and 2 of the EEC

PRESENTATIONS OF RESULTS IN SCIENTIFIC CONGRES

171.presentations in scientific meetings as author or co-author.

INVITED OR PLENARY CONFERENCES OR LECTURES.

75 presentations in scientific meetings under invitation

MAIN SUBJECTS OF RESEARCH

The Cellular Biochemistry and Biology Laboratory is working on cellular activation and interactions either in physiological conditions like ageing or in pathological situations in the vascular pathologies

Cellular Ageing mechanism has been investigated based on the in vitro model of cell culture. Modelisation of the ageing process has been possible using the thermodynamics of open systems; the role of Free radical and the importance of the antioxidant enzymeshave been deeply analysed.
Actual work is going on the molecular modulation of the genes expression during natural in vitro ageing and the stress induced ageing.The work compares the mRNA of specific genes overexpressed in old and stressed cells. It also investigates which signal transduction and transcriptional factor are activated durin the stress induced ageing

Study of endothelial cells under hypoxia in correlation with the development of varicose diseases.
We are studying the effects of hypoxia on endothelial cell metabolism either in vitro or in situ using umbilical and saphens perfused veins and the consequences of these modifications on the interactions between these cells and polymorphonuclear neutrophils and smooth muscle cells. The effect of phlebotonic drugs has been tested on cells in culture, on isolated organs and a clinical assay has been performed. This research is aimed to define the mechanism of varicose veins.

Development of new diagnostic assays using bioluminescence: ELISA, DNA probes for virus and bacteria detection.

Development of the biochips technology for the pharmaceutical, diagnostic and alimentation

PUBLICATIONS

The author's scientific output consists of 175 research papers in peer-reviewed international journals